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The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection

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Abstract

Colibacillosis results from infection with avian pathogenic *Escherichia coli* bacteria. Healthy broilers are resistant to inhaled *E. coli*, but previous infection with vaccine or virulent strains of Infectious Bronchitis Virus (IBV) predisposes birds for severe colibacillosis. We investigated whether IBV affects recruitment and function of phagocytic cells and examined NO production, phagocytic and bactericidal activity, and kinetics of peripheral blood mononuclear cells (PBMC) and splenocytes. Moreover, we measured cytokine mRNA expression in lung and spleen samples. Broilers were inoculated with IBV H120 vaccine or virulent M41 and challenged 5 days later with *E. coli* 506. A PBS control and *E. coli* group without previous virus inoculation were also included. Birds were sacrificed at various time points after inoculation (h/dpi). Inoculation with IBV induced extended and more severe colibacillosis than with *E. coli* alone. At 4 dpi, the number of KUL-01$^+$ PBMC in all *E. coli*-inoculated groups was significantly higher than in PBS-inoculated birds, which correlated with lesion scores. From 1 to 4 dpi, NO production by PBMC from all *E. coli*-inoculated animals was elevated compared to PBS birds. Bactericidal activity of PBMC in IBV-inoculated animals at 7 dpi was lower than in PBS- and *E. coli*-inoculated birds, but phagocytic capacity and recruitment were not severely impaired. In spleen samples of IBV-infected animals reduced expression of IL-1β, IL-6, IL-8, IL-10, IL-18 and IFN-γ mRNA was found 1 dpi. Our results suggest that enhanced colibacillosis after IBV infection or vaccination is caused at least by altered innate immunity and less by impairment of phagocytic cell function.

Keywords: IBV vaccine; Colibacillosis; Phagocytosis; Bactericidal activity; Cytokines; Innate immunity

1. Introduction

Predisposition for bacterial infections in the course of respiratory viral infections is found in various species, both avian and mammalian. In chickens, turkeys and ducks colibacillosis is often observed secondary to infection with respiratory agents e.g. Infectious Bronchitis Virus (IBV), Newcastle Disease Virus, and *Mycoplasma gallisepticum* (Igbokwe et al., 1996; Nakamura et al., 1994). In mice, influenza virus

Abbreviations: dpi, days post *Escherichia coli* inoculation; EID_{50}, median embryo infective dose; IBV, Infectious Bronchitis Virus; i.t., intratracheal; RPE, R-phycoerythrin.

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increases susceptibility to *Streptococcus pneumoniae* infection (Seki et al., 2004; Speshock et al., 2007) and measles virus predisposes for a range of bacterial infections such as *Listeria monocytogenes* (Slifka et al., 2003). In cattle, susceptibility for pneumonic pasteurellosis is greatly enhanced by a variety of respiratory viruses including bovine respiratory syncytial virus (Liu et al., 1999), bovine herpes virus-1 (Leite et al., 2002) and bovine coronavirus (Storz et al., 2000). In man, pneumonia has been a leading cause of death during influenza pandemics, supporting the widely held view that influenza virus predisposes for streptococcal infections (McCullers, 2006).

The mechanisms behind enhanced susceptibility to bacterial superinfection after viral infection have been studied extensively (Beadling and Slifka, 2004; Hament et al., 1999) but are still not well understood. A first set of hypotheses suggests increased susceptibility due to tissue damage in the respiratory tract resulting in functional impairment. Three possible causes have been described as mechanisms for functional damage. Viral replication in the upper respiratory tract causes loss of cilia and ciliated cells (Bakaletz, 1995), decreased ciliary activity impairs mucociliary clearance (Wilson et al., 1996) and finally, damage to epithelium may provide more attachment sites for bacteria (Ahmer et al., 1999).

A second set of hypotheses suggests altered innate immune responses. Impairment of innate effector functions, i.e. adhesion and entry, phagocytosis, killing, nitric oxide (NO) and superoxide production have been suggested previously (Ficken et al., 1987; Naqi et al., 2001; Read et al., 1999). Changes in recruitment or function of macrophages and neutrophils after bacterial superinfections have been described in mice (Navarini et al., 2006; Slifka et al., 2003).

Modulation of other innate functions affecting induction and/or control of adaptive responses might have a long term effect upon the host–pathogen interaction. Type I interferons (IFNs I) induced after viral infection have an antiviral effect, but can be detrimental for induction of anti-bacterial responses (Navarini et al., 2006; O'Connell et al., 2004), whereas type II IFNs play an important role in bacterial infections (Shtrichman and Samuel, 2001).

Goren (1978) developed an experimental model in broilers that demonstrated enhanced susceptibility to *Escherichia coli* after inoculation with IBV. Moreover, not only a virulent strain (IBV M41) enhanced inflammatory reactions on superinfection with *E. coli*, but the mild IBV vaccine strain (IBV H120) widely used in the field also enhanced susceptibility in a very similar way (Matthijs et al., 2003).

In our study to investigate which mechanisms underlie enhanced susceptibility to colibacillosis after IBV infection or vaccination, we examined the dynamics of pathogens and of immunopathological changes (Matthijs et al., accepted for publication).

The aim of this study was to detect whether innate effector functions such as the recruitment of effector cells and function of phagocytic cells, measured by NO production and bactericidal capacity, had been affected after IBV infection. As an alternative explanation for the enhanced colibacillosis we also examined altered expression of cytokine mRNA in spleen and lung samples.

2. Materials and methods

2.1. Experimental chickens

Eighteen-day-incubated eggs originating from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the Department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) each with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 $m^3\ h^{-1}$. Broilers were fed a commercial ration containing 12.4 MJ of metabolically energy per kg and 19.5% crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75% of *ad libitum* intake to diminish leg disorders and hydrops ascites. Tap water was provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35 °C at day 1 to 20 °C at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20 °C. All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2. Inocula

The IB vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50%) per dose (Nobilis® IB H120; batch 041106A).

The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, the Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50}/1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use and contained at least $10^{3.0}$ EID_{50}/ml of H120 virus and $10^{4.6}$ EID_{50}/ml of M41 virus. The *Escherichia coli* strain 506 (O78; K80) was isolated from a commercial broiler (Van Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs et al. (2003) and was used at a concentration of $10^{7.6}$ CFU/ml.

2.3. Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age, all groups were inoculated oculo-nasally (one droplet of 0.05 ml per bird in each eye and nostril) and intratracheally (1 ml per bird): groups 1 and 2 received distilled water, group 3 received IBV H120 virus and group 4 IBV M41 virus. At 32 days of age, groups 2, 3 and 4 were intratracheally inoculated with 1 ml *E. coli* 506 culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intratracheally per bird. For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 virus and *E. coli* broth) as the H120 group, and group 4 (IBV M41 virus and *E. coli* broth) as M41 group.

2.4. Clinical and post-mortem examination

Clinical signs of IBV infection were determined 1, 2, 4 and 5 days after IBV inoculation and after *E. coli* inoculation just before euthanizing. A bird was recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs et al., 2003).

From each group, 5 broilers were electrocuted and blood collected at 0.5 h, 3 h and at days 1, 2, 4 and 7 after *E. coli* inoculation (hpi/dpi). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (1991) in the left and right thoracic air sac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Matthijs et al., 2005).

2.5. Flow cytometric analysis of cell composition

Peripheral blood, spleen and lung were collected from sacrificed birds. Spleen tissue was squeezed through a 70 μm mesh to prepare single cell suspensions. Splenocytes and PBMC were isolated by density gradient centrifugation for 20 min at $850 \times g$ using Ficoll-Hypaque (density 1.078), washed twice with PBS (Cambrex) and adjusted to 5×10^7 cells/ml in RPMI1640 medium supplemented with 10% FBS, 2 mM glutamax-I (Gibco) and 100 U/ml penicillin and streptomycin (Gibco).

Cell suspensions were fluorescently labeled for 30 min on ice with mAb against thrombocytes (mouse anti-chicken CD41/CD61:FITC, Serotec), monocytes (mouse anti-chicken monocyte/macrophage KUL-01:FITC, Southern Biotechnology Associates) and $CD8^+$ cells (mouse anti-chicken CD8α:FITC, mouse anti-chicken CD8β:RPE, Southern Biotechnology Associates). Subsequently, cell-associated fluorescence was analyzed by flow cytometry using Cell Quest software (Becton Dickinson). Results are expressed as percentages of total viable cells.

2.6. Nitric oxide production assay

Cells were seeded in flatbottom 96-well plates at 2.5×10^6 cells in 50 μl culture medium (RPMI1640 + 2% chicken serum + 2 mM glutamax-I (Gibco) and 100 U/ml penicillin and streptomycin (Gibco). Cells were incubated for 48 h at 41 °C (5% CO_2) with 100 μl of either culture medium to determine background production of nitric oxide (NO), medium supplemented with 10 μg/ml heat-killed *E. coli* 506, or medium with 10 μg/ml *E. coli* LPS (from *E. coli* O55:B5, Sigma) to measure maximum NO production. After incubation, 50 μl/well of culture supernatant was transferred to sterile flatbottom 96-well plates. An NO dilution series (200–3.13 μM) was included on each plate as a standard curve to determine the amount of NO produced. To each well, 50 μl of Griess reagent (1% sulfanilamide and 0.3% naphtylenediamine, 1:1 in 2.5% phosphoric acid) was added, plates were incubated for 10 min at room temperature on a plate shaker and absorbance was recorded at 550 nm. For each chicken, NO secretion was calculated as total amount of NO produced by the cells after stimulation with heat-killed *E. coli* or *E. coli* LPS, minus the background NO production by unstimulated cells.

2.7. Phagocytosis and killing assay

Splenocytes and PBMC were incubated for 30 min at 10^8 cells in 1 ml RPMI 1640, 5% FBS containing 10^9 *E. coli* bacteria, in roundbottom 12 ml polypropylene

tubes (Greiner Bio-One) at 37 °C, 5% CO_2, to allow bacterial adhesion and entrance. At this point, gentamycin (200 μg/ml; Gibco) was added to each tube to kill extracellular bacteria and cells were incubated for an additional 30 min at 37 °C. Cells were washed once with PBS to remove the gentamycin, and either lysed directly with 1% saponin ($T = 0.5$ h) to release intracellular bacteria, or incubated for 14 h or 24 h in RPMI 1640 medium supplemented with 5% FBS and 20 μg/ml gentamycin before cell lysis. The cells were cultured in the presence of gentamycin to prevent re-infection and unlimited growth in the medium of *E. coli* released from dead cells. After saponin treatment, cell lysates were centrifuged and pellets were resuspended in 200 μl PBS and plated in duplicate (100 μl lysate per plate) on McConky Agar (MCA) plates (Biotrading) to determine the number of viable bacteria. The MCA plates were incubated overnight at 37 °C and colonies were counted blindly. Results were calculated as the sum of colonies on the duplicate plates. If the sum of colony counts on the duplicate plates was below 100 colonies, cells were considered successful in clearing the *E. coli*, whereas cells were considered unable to clear the bacteria when counts were over 300 colonies.

2.8. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples isolated from spleen and lung were screened for mRNA encoding IL-1β, IL-4, IL-6, IL-8, IL-10, IL-18, IFNα, IFNβ, and IFNγ. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Purified RNA was eluted in 30 μl RNase-free water and stored at −80 °C. Reverse transcription was performed using iScript cDNA Synthesis Kit (Biorad). Primers and probes (Applied Biosystems) were designed according to Kaiser et al. (2003) and Rothwell et al. (2004) and are listed in Table 1. For the quantitative RT-PCR assay TaqMan Universal PCR Master Mix (Applied Biosystems) was used. Primers were used at 300 nM and probes at 100 nM concentration. Amplification and detection of specific products was achieved with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) with the following cycle profile: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 59 °C for 1 min. Results were expressed as fold changes between samples, relative to the PBS group (Philbin et al., 2005). To correct for variation in RNA preparation and sampling, Ct values for cytokine-specific product for each sample were standardized using the Ct value of 28S-specific product for the same sample. To normalize RNA levels between samples within an experiment, the mean Ct value for 28S-specific product was calculated by pooling Ct values of all samples in that experiment. Well-to-well variations in Ct value of 28S-specific product about the experimental mean were calculated. The slope of the 28S dilution series regression lines was used to calculate differences in input of total RNA. Fold differences in cytokine expression between treatment groups were calculated relative to the reference ribosomal RNA, using a method adapted from Philbin et al. (2005). Fold differences were calculated from the C_T values C (for the cytokine) and C′ (for ribosomal RNA) using the equation $^{10}\log R_{(A/B)} = [(C_A - C_B)/S] - [(C'_A - C'_B)/S']$, where S and S' are, respectively, the slopes of plots of C_T value against the ^{10}logarithm of concentration for serial dilutions of cytokine DNA and ribosomal RNA, assayed on the same plate. This calculation avoids assumptions about the efficiency of the PCR amplifications and reduces to the common $\Delta\Delta C_T$ method in the case that both have perfect efficiency.

2.9. Statistical analysis

Between-group differences per time point were non-parametrically analyzed for each assay using the Mann-Whitney test. Partial correlations between different assays were determined per time point, with treatment as a control variable. Analysis was performed using the SPSS program and the probability level for significance was taken as $P < 0.05$.

3. Results

3.1. Clinical and macroscopical observations

Five days after IBV inoculation, all broilers in the M41 group and 10% of the broilers in the H120 group showed nasal discharge. Birds were examined macroscopically for colibacillosis lesions (Fig. 1). No lesions were observed in birds of the *E. coli* group at 0.5 h and 3 h after *E. coli* inoculation, at these time points all broilers of the H120 and the M41 groups, except one in the H120 group, had airsacculitis. Airsacculitis (colibacillosis lesions in the thoracic air sacs) was observed in chickens of all *E. coli*-inoculated groups between 1 and 4 dpi. At 7 dpi, no signs of colibacillosis were observed anymore in the *E. coli* group whereas all birds of the H120 and the

Table 1
Real-time quantitative RT-PCR primers and probes

RNA target	Probe/primer sequence (5′–3′)	Accession number
28S		
Probe	(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)	X59733
F primer	GGCGAAGCCAGAGGAAACT	
R primer	GACGACCGATTTGCACGTC	
IL-1β		
Probe	(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)	AJ245728
F primer	GCTCTACATGTCGTGTGTGATGAG	
R primer	TGTCGATGTCCCGCATGA	
IL-4		
Probe	(FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)	AJ621735
F primer	AACATGCGTCAGCTCCTGAAT	
R primer	TCTGCTAGGAACTTCTCCATTGAA	
IL-6		
Probe	(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)	AJ309540
F primer	GCTCGCCGGCTTCGA	
R primer	GGTAGGTCTGAAAGGCGAACAG	
IL-8		
Probe	(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)	AJ009800
F primer	GCCCTCCTCCTGGTTTCAG	
R primer	TGGCACCGCAGCTCATT	
IL-10		
Probe	(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)	AJ621614
F primer	CATGCTGCTGGGCCTGAA	
R primer	CGTCTCCTTGATCTGCTTGATG	
IL-18		
Probe	(FAM)-CCGCGCCTTCAGCAGGGATG-(TAMRA)	AJ276026
F primer	AGGTGAAATCTGGCAGTGGAAT	
R primer	ACCTGGACGCTGAATGCAA	
IFNα		
Probe	(FAM)-CTCAACCGGATCCACCGCTACACC-(TAMRA)	U07868
F primer	GACAGCCAACGCCAAAGC	
R primer	GTCGCTGCTGTCCAAGCATT	
IFNβ		
Probe	(FAM)-TTAGCAGCCCACACACTCCAAAACACTG-(TAMRA)	X92479
F primer	CCTCCAACACCTCTTCAACATG	
R primer	TGGCGTGCGGTCAAT	
IFNγ		
Probe	(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)	Y07922
F primer	GTGAAGAAGGTGAAAGATATCATGGA	
R primer	GCTTTGCGCTGGATTCTCA	

M41 groups had airsaccullitis. Systemic lesions (perihepatitis / pericarditis) were observed in 1 bird of the H120 group and 2 birds of the M41 groups at 7 dpi. No macroscopical signs of colibacillosis were observed in birds of the PBS group throughout the course of the experiment. More detailed clinical data can be found in Matthijs et al. (submitted for publication).

3.2. Flow cytometric analysis of blood mononuclear cells and splenocytes

The frequencies of thrombocytes, monocytes/macrophages, $CD8\beta^+$ and $CD8\alpha^+$ cells were analyzed as percentages of total viable cells in PBMC and splenocyte cell suspensions by flow cytometry at 1, 2, 4 and 7 dpi. Thrombocytes accounted for 60–75% of

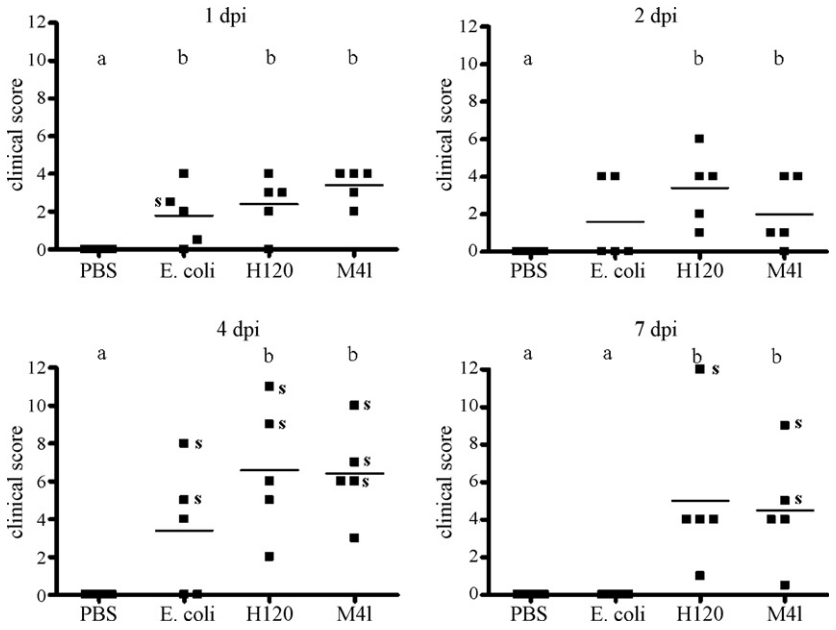

Fig. 1. Colibacillosis lesion scores at different time points after *E. coli* inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before *E. coli* inoculation, whereas the PBS group received no IBV or *E. coli*. Per treatment group, five birds were sampled at each time point. Each dot represents the total lesion score of an individual bird with a maximum score of 12. The horizontal lines indicate the mean scores of each treatment group. Birds with systemic signs of colibacillosis, characterized by lesions in liver and/or pericardium, are designated with 's'. Groups with different letters are significantly different ($P < 0.05$).

PBMC and 2–4% of splenocytes, with no significant differences between the treatment groups or fluctuations in time (data not shown). The percentage monocytes in peripheral blood of all *E. coli*-inoculated birds increased up to 4 dpi, and at 7 dpi, dropped to levels found in the PBS group (Fig. 2). A significant positive correlation ($P < 0.01$) between monocyte percentages and colibacillosis lesion scores was found at 4 dpi. Whereas an effect of the *E. coli* inoculation on monocyte frequencies was apparent, monocyte frequencies were not significantly decreased or increased in IBV-inoculated birds when compared to birds that received *E. coli* only. In the spleen at 1 dpi, a significantly higher percentage of macrophages was found in IBV-infected birds, and only in the H120 group, a higher percentage of macrophages was found at all time points. These changes were not significantly correlated to lesion scores. Significant variations in the

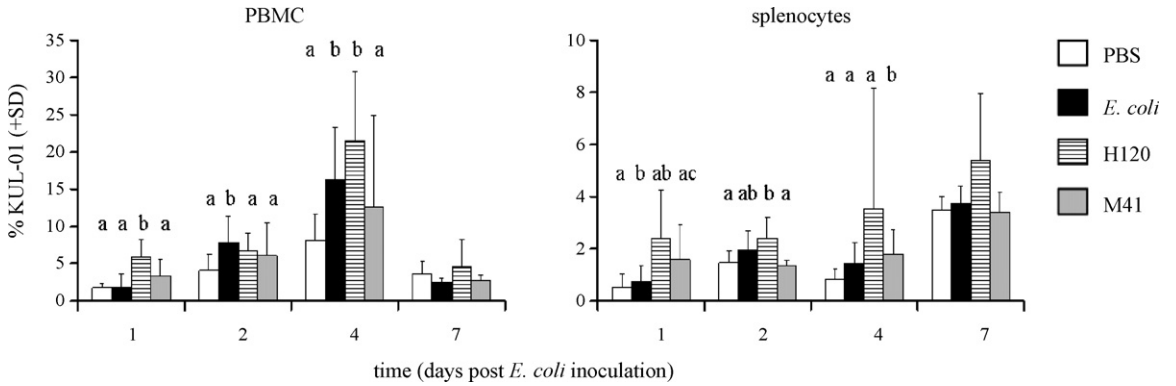

Fig. 2. Percentage ± S.D. of monocytes/macrophages (KUL-01) in PBMC and splenocytes at different time points after *E. coli* inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before *E. coli* inoculation, whereas the PBS group received no IBV or *E. coli*. Bars represent the average frequency of labeled cells per treatment group ($n = 5$), as a percentage of total live cells. Groups with different letters are significantly different ($P < 0.05$).

percentages of CD8β$^+$, PBMC and splenocytes between the treatment groups were observed at different time points, but these variations were not related to an effect of either *E. coli* or IBV (data not shown). Similar fluctuations in the percentages of CD8α$^+$β$^-$ cells were found, apart from 1 dpi, where the M41 group showed higher percentages of CD8α$^+$β$^-$ cells than the other groups, but no significant correlation was found with the *E. coli* or IBV inoculation (data not shown).

3.3. Nitric oxide production

The Griess reaction assay was used to determine whether IBV inoculation had an effect on the production of NO by splenocytes and PBMC after subsequent stimulation with heat-killed *E. coli* bacteria or *E. coli* LPS. NO production by PBMC of the *E. coli*-inoculated groups at 1 dpi was significantly higher than of the PBS group (Fig. 3). Furthermore the IBV-inoculated groups, most notably the H120 group, showed higher NO production than the *E. coli* group, but at 2 dpi, NO production of the IBV-inoculated groups almost halved. At 7 dpi, NO production of the *E. coli* group had dropped to the level of the PBS group, whereas NO production by both the H120 and M41 groups was elevated compared to the PBS and *E. coli* groups.

NO production by PBMC and splenocytes after *E. coli* LPS-stimulation showed a pattern comparable to stimulation with heat-killed *E. coli*, although the amount of NO produced was approximately twice as high (data not shown).

3.4. Phagocytosis and killing assay

The ability of PBMC and splenocytes to kill *E. coli* bacteria after internalization was determined at 4 and 7 dpi, based on the number of colonies found after 24 h incubation. Uptake of bacteria was confirmed after 14 h incubation as the number of *E. coli* colonies retrieved from the PBMC and splenocytes and was higher than 300 in all chickens (data not shown). At 7 dpi, in the H120 and M41 groups the PBMC of 3 respectively 4 out of 5 chickens did not kill engulfed bacteria based on high colony counts, whereas only 1 chicken in the PBS group and 1 chicken in the *E. coli* group did not kill intracellular bacteria (Table 2). At 4 dpi, splenocytes from all groups successfully killed *E. coli* bacteria. At 7 dpi however, splenocytes from 2 chickens in the H120 group were not able to kill the bacteria within 24 h incubation (data not shown).

3.5. Real-time quantitative RT-PCR

To examine the effects of IBV and *E. coli* inoculation on systemic immune functions, cytokine mRNA expression of spleen and lung samples were measured by real-time quantitative RT-PCR. No significant differences between the groups were found at any time point for IL-4, IFNα and IFNβ in either spleen or lung samples (data not shown). At 1 dpi, spleen mRNA levels for IFNγ and the pro-inflammatory cytokines IL-1β and IL-6 (data not shown) as well as IL-8, IL-18 and IFNγ (Fig. 4A) were significantly upregulated in the *E.*

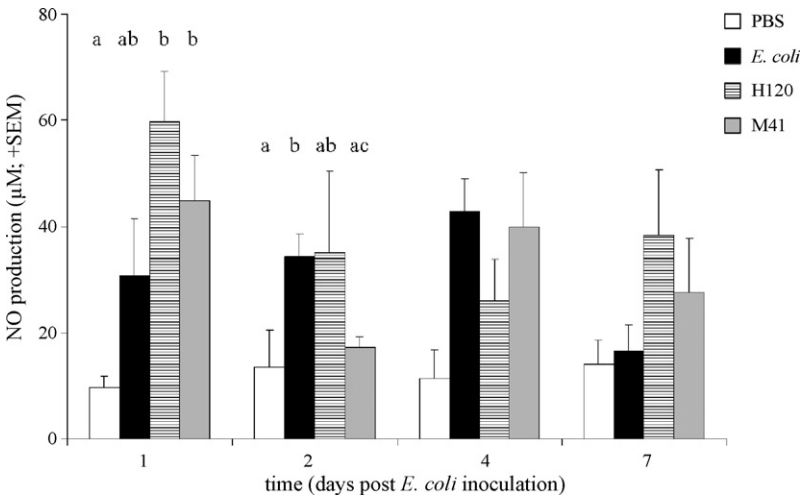

Fig. 3. Means ± S.E.M. of *E. coli*-induced NO production in response to heat-killed *E. coli* 506 by PBMC per group, at various time points after *E. coli* inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before *E. coli* inoculation, whereas the PBS group received no IBV or *E. coli*. NO production by cells of individual chickens is calculated as the total NO production after stimulation with heat-killed *E. coli* or LPS, minus the background NO production of unstimulated cells. Groups with different letters are significantly different ($P < 0.05$).

Table 2
Phagocytosis and killing of bacteria by PBMC of chickens killed 4 and 7 days after *E. coli* inoculation (dpi)

Group	Colony counts 4 dpi[a]			Colony counts 7 dpi[a]		
	<100	100–300	>300	<100	100–300	>300
PBMC						
PBS[b]	3	0	2	0	4	1
E. coli	0	3	2	2	2	1
H120	0	2	3	1	1	3
M41	1	1	3	0	1	4

Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine strain H120 (group: H120), virulent IBV strain M41 (group: M41) or PBS (groups: PBS and *E. coli*) and 5 days later inoculated with *E. coli*, the PBS group with PBS. Each group consisted of five broilers. Numbers represent the number of chickens in each group displaying the specified killing efficiency. For each chicken, samples were run in duplicate.

[a] The number of colonies retrieved from phagocytic cells after lysis was used as a measurement for killing efficiency; retrieval of less than 100 colonies was considered successful clearance, whereas retrieval of more than 300 colonies was considered as a failure to clear *E. coli*.

[b] Inoculated at 32 days with glucose broth instead of *E. coli* broth.

coli-inoculated group in comparison to the IBV-inoculated groups. Similarly, mRNA levels for the anti-inflammatory cytokine IL-10 was increased significantly in *E. coli*-inoculated birds compared to the IBV-inoculated birds. The mRNA levels of these cytokines dropped sharply from 2 dpi onward. Interestingly, at 7 dpi, only in the H120 group, IL-6, IL-10 and IFNγ mRNA expression was still higher than the *E. coli* group. Tests were carried out to find whether altered expression of cytokine mRNA in IBV-inoculated birds was also found in lung samples (Fig. 4B). In lung samples, no significant differences between the groups were found at any time point for IL-18, IFNβ and IFNγ (data not shown). In contrast to our findings in the spleen, at 3 hpi, mRNA levels for IL-6, IL-8, IFNγ (data not shown), IL-10 and IL-1β (Fig. 4B) in lung samples of birds of the H120 and especially the M41groups were higher than those of the *E. coli* group. However, at 1 dpi, mRNA expression of these cytokines in the *E. coli* group had increased similar to, or higher than that of the birds in the IBV infected groups. Levels remained elevated at 2 dpi, with highest expression in the IBV H120 group. At 7 dpi, mRNA expression levels of all three *E. coli*-inoculated groups had dropped to the level of the birds of the PBS group.

4. Discussion

Enhanced susceptibility for bacterial infections as a consequence of viral infection is reported in varying combinations in various species (Hament et al., 1999). In poultry, severe colibacillosis after infections with

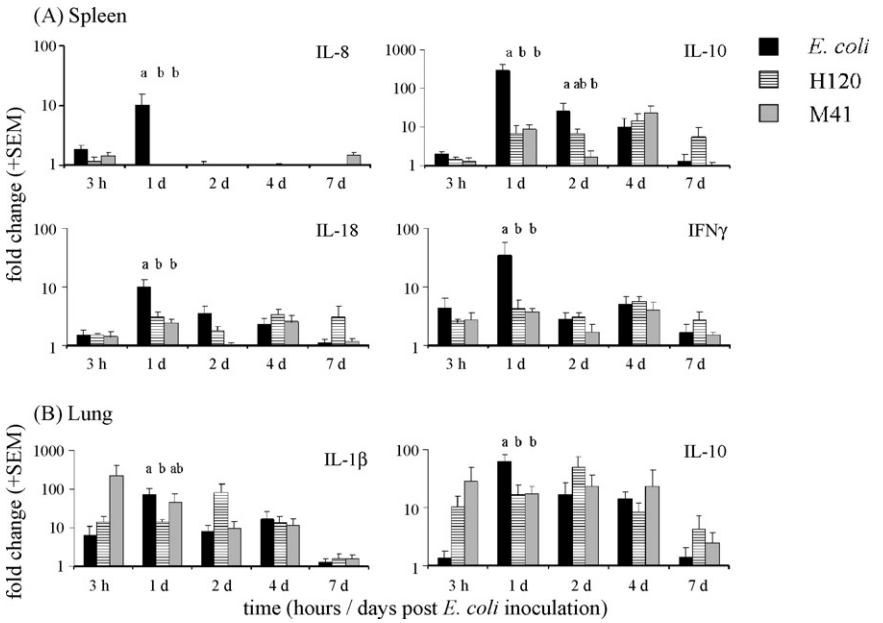

Fig. 4. Real-time quantification of cytokine mRNA expression by cells isolated from (A) splenocytes and (B) lung tissue samples of PBS chickens (white bars), chickens inoculated with *E. coli* only (black bars), IBV H120 + *E. coli* (hatched bars) and IBV M41 + *E. coli* (grey bars). Data are expressed as mean relative fold increase at different time points after *E. coli* inoculation, compared to samples of PBS birds. Error bars show S.E.M. for triplicate samples of five birds per treatment group. Groups with different letters are significantly different ($P < 0.05$).

Infectious Bronchitis Virus is well documented (Peighambari et al., 2000; Vandekerchove et al., 2004) and is a serious problem in commercial chickens.

Interestingly, not only the virulent IBV M41 field strain induces enhanced susceptibility to subsequent *E. coli* infection, but the mild H120 vaccine strain also induces this enhanced colibacillosis to about the same level (Matthijs et al., 2003). A model based on these findings was used to study the phenomenon and test the contribution of different possible factors causing the phenomenon. In a separate paper, we addressed the question whether local changes at the surfaces of the trachea, lung and airsacs might facilitate local bacterial growth and subsequent entry of bacteria. The results of that study showed that the clearance of the bacteria from the lung was comparable for all the groups inoculated with *E. coli*, irrespective of previous exposure to virus (Matthijs et al., submitted for publication).

In this paper we examined whether prior exposure to IBV virus (M41 or H120) affected the effector functions of mononuclear cells resulting in the phenomenon of enhanced susceptibility, a hypothesis supported by several papers (Ficken et al., 1987; Naqi et al., 2001; Slifka et al., 2003). For this question, NO production by mononuclear cells isolated from the spleen and PBMC were tested. After *E. coli* inoculation, the NO production in samples upon stimulation with heat-killed *E. coli* bacteria or *E. coli* LPS (data not shown) was strongly increased in all groups, irrespective of prior exposure to IBV, although at 1 dpi the increase was highest in IBV-inoculated birds. Another difference was observed at 7 dpi, where birds exposed to *E. coli* alone showed diminished NO production compared to birds also exposed to either IBV strain (M41 or H120). This is in line with the clinical picture of bacterial persistence in broilers previously exposed to IBV.

Subsequently, the internalization and intracellular killing of bacteria was tested in splenocytes and PBMC of all groups. Again all groups exposed to *E. coli*, irrespective of earlier exposure to IBV, responded similarly in the assay at 4 dpi, in that the cells had a diminished killing capacity. At a later time point (7 dpi), bactericidal capacity in the groups exposed to IBV was still lower than the bactericidal capacity of the group exposed to *E. coli* alone, suggesting a slower recovery. This once more was in line with the clinical recovery observed in the *E. coli* alone group.

The results of the NO production and of the internalization and intracellular killing of bacteria by the individual splenocytes or PBMCs suggest that the small changes in effector functions of those cells cannot be the only reason for the phenomenon of enhanced colibacillosis in animals previously exposed to virus. However, changes in the number of cells and/or their recruitment could still influence the outcome of the secondary infection. We therefore evaluated the percentage of effector cells by FACS analysis. Major differences that could explain the enhanced colibacillosis in the samples of the dual infected groups were not found.

Because no major changes in the effector functions of the mononuclear cells could be found to explain the phenomenon of enhanced colibacillosis, other mechanisms to explain the findings were sought. Supported by the observation that clinical colibacillosis seemed mainly the result of a systemic effect, and not directly of a local lung condition, we set out to find other explanations and looked at whether cytokine profiles would clarify the clinical picture. This might lead to insights into how the innate capacity of the animals was modulated at the time of *E. coli* exposure. Therefore, we examined mRNA expression in splenocytes and in samples of the lung.

After *E. coli* inoculation alone, the pro-inflammatory cytokines IL-1β and IL-6 were strongly increased in both splenocytes and lung tissue. This was expected, because both cytokines play an important role in initiating an acute-phase immune response against invading pathogens and activating a wide range of immune cells such as macrophages and T-cells (Wigley and Kaiser, 2003).

The pro-inflammatory chemokine IL-8 was found to be upregulated in lung samples as early as 3 hpi, and in splenocytes at 1 dpi. The findings in the lung are in agreement with its function as a chemotractant, produced at the infection site in order to recruit heterophils and initiate a rapid local inflammatory response (Withanage et al., 2004). The upregulation in spleen samples was only found in the *E. coli* group and is likely due to the fact that the bacteria are not retained in the respiratory tract but spread systemically. Surprisingly, in the IBV groups no IL-8 mRNA is found in the spleen, which might suggest that a lack of response in the spleen can contribute to prolonged colibacillosis. The mRNA expression of pro-inflammatory mediators IL-1β and IL-6 in the spleen at 1 dpi in IBV groups also lagged behind that of the *E. coli* group.

Not only the cytokines IFNγ and IL-18, but also IL-10 were found to be upregulated in splenocytes and lung samples of birds inoculated with *E. coli* alone. IFNγ is a potent macrophage-activating factor and IL-18 is an important inducer of IFNγ production (Wigley and Kaiser, 2003). Both IFNγ and IL-18 are strongly linked to a cell-mediated Th1-like immune response (Staeheli

et al., 2001). IL-10 on the other hand is a promotor of Th2-like immune responses, predominantly by inhibiting pro-inflammatory and Th1 cytokines (Rothwell et al., 2004), which can increase host susceptibility to bacterial diseases through its anti-inflammatory effects, including suppression of macrophage function. Previous studies in chickens showed that IL-10 production could be accompanied by either downregulation of IFNγ production (Abdul-Careem et al., 2007; Rothwell et al., 2004), but also by concurrent upregulation of IL-10 and IFNγ (Hong et al., 2006). Moreover, it should be noted that IL-10 is known to have immunostimulatory effects in certain species, inducing MHC class II upregulation and cytotoxic T-cell stimulation (Groux and Cottrez, 2003).

In our chicken model, we find that high mRNA expression of pro- and anti-inflammatory cytokines at 1 dpi in spleen samples is not found in the birds that were inoculated with IBV and *E. coli*, but only in the *E. coli* inoculated birds, and thus seems to be associated with recovery of colibacillosis. The differences in mRNA expression are consistent with the finding that virus-induced modulation of the immune response plays an important role in the susceptibility to subsequent bacterial infection.

In conclusion, we observed that both vaccine and virulent IBV caused enhanced colibacillosis, but IBV did not significantly affect phagocytic capacity and NO production of peripheral mononuclear cells and splenocytes. However, IBV did alter the systemic cytokine mRNA expression patterns after *E. coli* inoculation in commercial broilers likely resulting in enhanced colibacillosis.

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